AD_____

Award Number: DAMD17-00-1-0144

TITLE: Mechanisms of Mutation in Non-Dividing Cells

PRINCIPAL INVESTIGATOR: Doctor Joseph F. Petrosino
Susan Rosenberg

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: May 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Annual (15 Apr 2001 - 14 Apr 2002)

4. TITLE AND SUBTITLE
Mechanisms of Mutation in Non-Dividing Cells

5. FUNDING NUMBERS
DAMD17-00-1-0144

6. AUTHOR(S)
Doctor Joseph F. Petrosino
Susan Rosenberg

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail:jp691032@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract Understanding how mutations arise in non-growing cells will help illuminate mechanisms of oncogenesis, tumor progression, and resistance to chemotherapeutic drugs. To this end, I have been addressing how antibiotic resistance mutations occur in non-, or slowly-growing enterobacteria cells. Previously, our laboratory discovered that RecA (an hRAD51 homolog) and RecBCD recombination repair proteins are necessary for the acquisition of ß-lactam drug-resistant mutations in the *Escherichia coli* chromosome during stationary-phase. The data suggest that either the SOS DNA damage-repair response, recombinational DNA repair, or both, are involved in the mutation pathway. I have improved the *E. coli*-based system to examine the genetic and biochemical processes involved in this mutational mechanism in detail. Initial results in this improved system suggest that ß-lactam resistance mutations occur not only in a growth-dependent manner but also in response to stress induced by starvation. The starvation conditions may mimic the environment pathogens or tumor cells may encounter in inflicted patients under stress, chemotherapeutic treatment, or other anti-tumor drug regimen in which cells are in a state of slow-, or non-growth. In addition, I have engineered a reporter construct that will allow me to enrich for those cells undergoing stationary-phase mutation so I may study the genetic and biochemical intermediates involved in this mutation mechanism.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	16
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

When cancers develop in tissues of non-dividing or slowly growing cells, the first cancerous cell must escape growth regulation and acquire mutations to become transformed. Mutational mechanisms specific to non-growing cells may facilitate this progression from quiescence to division. Similarly, when tumors develop resistance to chemotherapeutic drugs, such as mitotic inhibitors, they acquire mutations and other changes that allow growth in the presence of the drugs. Moreover, the growth stage when mutation is most active is not known. G_0, the analog of bacterial stationary phase, could be an important period for spontaneous mutation. To understand these processes, it is important to elucidate the mechanism(s) of mutation in non-dividing and slowly growing cells.

A mutation mechanism has been described in stationary-phase *Escherichia coli* that requires recombination proteins, and produces sequences different from growth-dependent mutations (7, 9, 10, 19, 20). Stationary-phase mutation is best understood in the *E. coli* Lac assay in which cells with a *lac* +1 frameshift allele on an F' plasmid generate Lac^+ mutants upon starvation on lactose medium (2). The stationary-phase mutations, but not growth-dependent Lac^+ mutations, require recombination proteins (RecA, RecBCD, RuvABC) and the bacterial SOS DNA damage-repair response (5, 9, 10, 14). Furthermore, DNA mismatch-repair protein MutL is transiently limited during stationary-phase mutation (8, 13). Recently, our lab determined that error-prone DNA polymerase IV (encoded by *dinB,* the ortholog of DINB1 in humans) is the SOS component required for Lac^+ stationary-phase mutation (15).

The *E. coli* recombination and DNA repair proteins important for stationary-phase mutation have human homologs implicated in breast and other types of cancer. For example, Rad51, a homolog of *E. coli* RecA, interacts with breast cancer tumor suppressor proteins BRCA1, and BRCA2 as well as the p53 tumor suppressor protein (16, 21, 22). Mutations in these proteins are associated with predisposition to breast cancer. In addition, defects in human homologs of the *E. coli* mismatch repair proteins MutS and MutL underlie hereditary nonpolyposis colorectal cancer (HNPCC).

Mutation mechanisms that occur in non-dividing or stationary-phase cells may generate ß-lactam antibiotic resistance mutations. ß-lactam antibiotics kill actively dividing bacteria, and so those cells that are not dividing have an opportunity to acquire resistance mutations *via* stationary-phase mutation. Chromosome-encoded AmpC ß-lactamases inactivate specific ß-lactam antibiotics and are widespread in enterobacteria. *E. coli* carries all of the genes required for *ampC* production except for *ampR*, the transcriptional activator of *ampC*. Loss-of-function mutations in *ampD*, an indirect regulator of *ampR*, are found in ß-lactam resistant pathogens and cause resistance in *E. coli* when the Enterobacter *ampRC* genes are expressed from a plasmid (12). The focus of this project is on the mechanisms of chromosomal *ampD* mutation in stationary-phase *E. coli* to improve the understanding of how antibiotic resistance mutations can arise in quiescent cells.

Recently, our lab demonstrated stationary-phase mutation occurring directly on the *E. coli* chromosome (1). However, some targets on the *E. coli* chromosome are less prone than others to stationary-phase mutation (18). The reasons for this observation are not clear and probably relate to the fundamental mechanism underlying stationary-phase

mutation. Many aspects of the mutation processes involved may be relevant to how oncogenic mutations occur in non-dividing cells in humans, and how tumors treated with drugs that kill dividing cells may still acquire mutations that enable the drugs to be evaded.

Body

Year 1 Key Research Accomplishments (items 1-5 published in *Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1):

- The *ampRC* genes were integrated into the *E. coli* chromosome.
- Mutant alleles of the *recA, recB, recG, lexA3, sulA, sulA lexA51,* and *dinB* recombination and SOS genes were incorporated into *ampRC*-containing cells.
- Growth-dependent and stationary-phase mutation were quantified using this system.
- Stationary-phase, but not growth-dependent, mutation requires *recA* and increases in *recG*-deficient cells.
- 20 out of 20 growth-dependent Amp^R mutations in rec^+ and *recA* cells occurred in *ampD* as determined by plasmid complementation and sequence analysis.
- A *sulA* promoter-*gfp* fusion was constructed and integrated into the *E.coli* chromosome to isolate SOS-induced cells.
- The *sulA* promoter-*gfp* fusion was demonstrated to be LexA-regulated as intended.

Response to first year summary technical issues:

The reviewer of the year one summary made several interesting and insightful comments that are addressed here.

Technical issues:

a) The reviewer commented on an apparent contradiction in mutation results reported in the *recA* background.--Fluctuation tests were done in $RecA^-$ cells to show that recombination functions are not required for growth-dependent, spontaneous mutations in *ampD*. Spontaneous mutations are traditionally related to strand-slippage events and polymerase-errors and are not perceived to require recombination functions, such as those encoded by *recA*. These fluctuation tests were performed partly as proof-in-principle experiments for the chromosomal *ampRC* system, as well as to confirm our presumption that little or no recombination-dependent spontaneous mutation is occurring in these cells.

The other mutation studies demonstrating *recA* dependence relate solely to mutation happening in stationary-phase cells after they are starved in the presence of lactose. In studies performed to date by our lab and others, mutation in stationary-phase cells is strikingly different to mutation in growing cells. One of the fundamental mechanistic differences is the requirement for recombination functions in stationary-phase, but not spontaneous (i.e. growth-dependent), mutation .

b) The reviewer questioned how roles for the RecA and RecG proteins in stationary-phase mutation are assigned, as well as how will we determine which function(s) of the multi-purpose RecA protein is important for stationary-phase mutation.--Various genetic

requirements for stationary-phase mutation were determined in experiments where strains isogenic to the parent *ampRC*$^+$ *E. coli* were deleted for genes of interest (e.g. *recA* and *recG*). It was found that *recA* is absolutely required for *ampD* stationary-phase mutation, and deletion of the *recG* gene leads to a greater frequency of *ampD* mutation. As a result, *recA* is believed to be a primary component of the stationary-phase mutation mechanism, while the role of *recG* is not clear.

As the reviewer points out, RecA is also a critical component of the bacterial SOS DNA damage-repair response. To discern which functions of RecA are required for stationary-phase mutation, genes specific to RecA-mediated DNA recombination/repair and genes belonging to the SOS response were deleted in isogenic *ampRC*$^+$ strains and tested for their ability to undergo stationary-phase *ampD* mutation. It was found that both recombination and the SOS-induced *dinB* gene are required for mutation in this system (described below in **Year 2** results). (DinB, or DNA polymerase IV, is an error-prone DNA polymerase which has homologs in all three domains of life [archaea, bacteria, and eukaryotes—including humans].) Therefore, it appears both the SOS inducing- and recombination functions of RecA are required for stationary-phase *ampD* mutation.

c) The reviewer brought up complications with working with a starvation model for studying stationary-phase mutation, particularly the presence of free radicals, which may promote stress.--The role of damaging radicals and DNA base-analogs which may be present in higher quantities during starvation and/or stationary phase is of great interest to our laboratory in both the context of the original Lac system as well as our *ampRC* system and, of course, in eukaryotic cells. Preliminary Lac stationary-phase mutation studies have been initiated (by others in our lab) with strains deleted for genes involved in the repair of oxidative damage in the cell. These studies have generated potentially interesting results that need to be pursued in both the Lac and *ampRC* systems. The relevance to breast/eukaryotic cancer systems will be explored as well.

Year 2

The genetic requirements for ß-lactam resistance mutation was studied further in stationary phase cells starving on lactose. Among other interesting results, I found that DNA PolIV is required for stationary-phase ampicillin resistance mutation as it was found to be for Lac stationary-phase mutation. When the *dinB* gene is interrupted by a kanamycin-resistance gene cassette, mutation to ampicillin resistance drops to background levels in cells starving on lactose. Recent papers suggest the possible involvement of three of the four DinB (PolIV)/UmuDC superfamily polymerases present in vertebrates in somatic hypermutation of the immunoglobulin genes (3, 17, 23).

I have also found that stationary-phase ß-lactam resistance mutation requires the DNA recombination protein *recB* which, when mutated, eliminates the RecBCD double-stranded DNA (dsDNA) helicase/exonuclease. RecBCD normally prepares DNA for recombination by unwinding dsDNA and loading RecA protein onto the newly created ssDNA to prepare it for recombination (4). The requirement of RecBCD suggests that DNA double-strand ends (DSEs) are an intermediate in the stationary-phase mutation mechanism. How these DSEs are generated is of great interest and is currently under

investigation. Damage from free radicals, as mentioned by the reviewer from my year one report, is one hypothesis that we are testing.

In year one, we analyzed growth-dependent *ampD* mutant sequences to ascertain the sequence spectrum of growth-dependent mutation in our system (Appendix 1). In year two, I began sequencing *ampD* mutants that were isolated from our stationary-phase mutation experiments to compare the sequence spectra of growth-dependent and stationary-phase mutation in our system. So far, I have collected 20 *ampD* stationary-phase mutant sequences and have found that the sequence spectrum appears significantly different in stationary-phase, compared to that of growth-dependent, mutants. Hotspots (where mutations seem to cluster) in the growth-dependent mutants, were non-existent in the stationary-phase mutants. Also, there is a high proportion of substitution mutations in the stationary-phase mutants compared to the growth-dependent mutants. Among the substitution mutations, I found a high-proportion of G to T transversions. Interestingly, G-C to T-A transversions were found to be increased nearly 100-fold when PolIV is overexpressed in *E. coli* (11). Also, G to T transversions are found in cells unable to repair 8-oxyguanine oxidative damage. This sequence information therefore points to/supports two potential pathways leading to mutation: one through PolIV(already shown through genetic requirement testing), and one possibly through oxidative damage. The role of oxidative damage in the stationary-phase mutation pathway can be tested by deleting genes required for the repair of 8-oxyguanine damage (e.g. *mutY*—such experiments are underway in our laboratory), however the results of such experiments have to be carefully interpreted and followed up, because as the reviewer from year 1 mentioned, primary and secondary causes and effects of this mutation process will be detected in our system.

We are currently conducting control experiments that will be necessary to present for publication of our findings. One major set of controls is reconstruction experiments to show that each of the ampicillin resistant mutants that arises is capable of growing in our test conditions in each of the genetic backgrounds we have examined. For example, it's possible that mutations in *ampD*, in a *recA* background, prohibit growth in our assay conditions and is what causes the decreased mutation phenotype we observe. To eliminate this possibility, we subject approximately 100 *recAampD* cells (or other relevant background) to the experimental conditions (i.e. starve on a lactose plate and overlay with ampicillin and glycerol after 4 days) and observe whether all, or only a fraction of, the cells plated are able to grow into colonies. This control is currently being repeated for each strain background I have examined.

Another control I need to perform is one to eliminate the possibility that the ampicillin resistant mutants that arise are not mutators (capable of mutation at higher frequencies than the rest of the bacterial population—often the result of a defect in mismatch repair). It has been shown that, in a given bacterial population, there always may be a small percentage of cells that are mutator (6). Simple mutation experiments with ß-lactam resistant mutants compared to ß-lactam sensitive cells will easily discern whether or not mutator populations are skewing our results. (Preliminary data suggest that they are not.)

Last year, I reported on a *sulA* promoter/*gfp* construct that will enable us to enrich for starving, stationary-phase cells undergoing an SOS response from cells which are not at different times during experimental trials. It is expected that the sorted cells that are

green will demonstrate hypermutability to ß-lactam resistance and to Lac$^+$ compared to cells sorted against. The DNA and proteins in this subpopulation can be purified to further examine the changes and signals occurring in these cells during stationary-phase mutation. Over the past year, I performed several follow-up experiments with these cells to help me ascertain whether future sorting experiments will be successful. In addition to the original *sulA* promoter/*gfp* construct, I have made recombination protein-deficient derivatives as controls and additional strains to test in our sorting experiments. These cells were examined by fluorescence microscopy to determine their level of SOS induction at the cellular level. It was found that certain recombination-deficient backgrounds have a higher fraction of cells induced for the SOS response compared to the recombination-proficient parent strain. However, never were all cells chronically SOS-induced. These observations suggest that our construct will enable us to sort subpopulations of SOS-induced cells from those not experiencing an SOS response.

Key Research Accomplishments:

- Additional alleles have been introduced into the *ampRC*$^+$ tester strain including *recB* and *ruvC*
- ß-lactam resistance mutation was shown to be PolIV, RuvC (Holliday Junction resolvase), and RecB dependent
- Sequence spectra of stationary-phase mutants shows a different mutation pattern compared to growth-dependent mutants (supports PolIV requirement and suggests possible role of oxidative damage in stationary-phase mutation)
- *gfp*- fusion studies show discrete levels of SOS induction within bacterial populations
- Control experiments leading to the publication of our stationary-phase mutation work are underway
- Paper describing the *ampRC*$^+$ system has been published (*Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1)

Reportable Outcomes:

Abstract submissions:

2002 Gordon Research Conference on Mutagenesis. July 28-August 2, Lewiston, Maine. *E. coli* DNA PolIV/DinB and recombination proteins are required for stress-induced antibiotic resistance mutations.

2002 Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, September 25-September 28, Orlando, Florida. Recombination proteins and the Polκ ortholog, PolIV are required for stationary-phase antibiotic resistance mutation.

2001 Molecular Genetics of Bacteria and Phages Meeting, July 31-August 5, University of Wisconsin, Madison. Resistance to ß-lactam antibiotics mediated by stationary-phase mutation.

Publications:

Petrosino J.F., Pendleton A.R., Weiner J.H., Rosenberg S.M. 2002. Chromosomal system for studying AmpC-mediated beta-lactam resistance mutation in Escherichia coli. Antimicrob Agents Chemother. **46**:1535-9.

Petrosino, J. F., Hastings, P. J., Rosenberg, S. M. (2001). RecBCD enzyme, pathway. Encyclopedia of Genetics. Sydney Brenner and Jeffrey H. Miller, Editors-in-chief. Academic Press, N.Y.

Conclusions

The genetic requirements observed for stationary-phase ß-lactam resistance mutation so far suggest that a similar mechanism underlies both the *ampRC*$^+$ and the Lac systems. However, *ampD* mutation takes place on the bacterial chromosome and not on the F' and differences between mutation in these two regions is currently being investigated. The *ampRC*$^+$ system provides a first look at the full sequence spectra of recombination/PolIV-dependent stationary-phase mutation. The F' plasmid-based Lac and chromosomal Tet^R systems specifically select for –1 frameshift mutations and therefore display a strong bias when looking at their respective sequence patterns. Data thus far suggest that *ampD* stationary-phase mutations are significantly different from *ampD* growth-dependent, spontaneous mutations. The data supports the role of PolIV as the polymerase generating the mutations, and also hints at the role of oxidative damage as a potential component of the mutational mechanism as well.

Preliminary experiments leading to flow-cytometry sorting of the hypermutable cell subpopulation show that DNA damage is constantly occurring in a fraction of a given cell population and that this fraction is different depending on the genetic background of the cells being observed. Actual sorting of the cells is scheduled for the upcoming year.

References:

1. **Bull, H. J., M.-J. Lombardo, and S. M. Rosenberg.** 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proc. Natl. Acad. Sci. USA. **(in press)**.
2. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics. **128:**695-701.
3. **Chicurel, M.** 2001. Can organisms speed their own evolution? Science. **292:**1824-1827.
4. **Churchill, J. J., D. G. Anderson, and S. C. Kowalczykowski.** 1999. The RecBC enzyme loads RecA protein onto ssDNA asymmetrically and independently of chi, resulting in constitutive recombination activation. Genes Dev. **13**(7)**:**901-11.

5.**Foster, P. L., J. M. Trimarchi, and R. A. Maurer.** 1996. Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. Genetics. **142:**25-37.
6.**Giraud, A., M. Radman, I. Matic, and F. Taddei.** 2001. The rise and fall of mutator bacteria. Curr. Opin. Microbiol. **5:**582-585.
7.**Harris, R. S., H. J. Bull, and S. M. Rosenberg.** 1997. A direct role for DNA polymerase III in adaptive reversion of a frameshift mutation in *Escherichia coli*. Mutat. Res. **375:**19-24.
8.**Harris, R. S., G. Feng, C. Thulin, K. J. Ross, R. Sidhu, S. Longerich, S. K. Szigety, M. E. Winkler, and S. M. Rosenberg.** 1997. Mismatch repair protein MutL becomes limiting during stationary-phase mutation. Genes & Dev. **11:**2426-2437.
9.**Harris, R. S., S. Longerich, and S. M. Rosenberg.** 1994. Recombination in adaptive mutation. Science. **264:**258-260.
10.**Harris, R. S., K. J. Ross, and S. M. Rosenberg.** 1996. Opposing roles of the Holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. Genetics. **142:**681-691.
11.**Kim, S. R., G. Maenhaut-Michel, M. Yamada, Y. Yamamoto, K. Matsui, T. Sofuni, T. Nohmi, and H. Ohmori.** 1997. Multiple pathways for SOS-induced mutagenesis in *Escherichia coli*: an overexpression of *dinB/dinP* results in strongly enhancing mutagenesis in the absence
of any exogenous treatment to damage DNA. Proc. Natl. Acad. Sci. USA. **94:**13792-13797.
12.**Lindberg, F., S. Lindquist, and S. Normark.** 1987. Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. J. Bacteriol. **169:**1923-1928.
13.**Longerich, S., A. M. Galloway, R. S. Harris, C. Wong, and S. M. Rosenberg.** 1995. Adaptive mutation sequences reproduced by mismatch repair deficiency. Proc. Natl. Acad. Sci. USA. **92:**12017-12020.
14.**McKenzie, G. J., R. S. Harris, P. L. Lee, and S. M. Rosenberg.** 2000. The SOS response regulates adaptive mutation. Proc. Nat. Acad. Sci. USA. **97:**6646-6651.
15.**McKenzie, G. J., P. L. Lee, M.-J. Lombardo, P. J. Hastings, and S. M. Rosenberg.** 2001. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell. **7**(3)**:**571-579.
16.**Patel, K. J., V. P. C. C. Yu, H. Lee, A. Corcoran, F. C. Thistlewaite, M. J. Evans, W. H. Colledge, L. S. Friedman, B. A. J. Ponder, and A. R. Venkitaraman.** 1998. Involvement of BRCA2 in DNA repair. Mol. Cell. **1:**347-357.
17.**Rogozin, I. B., Y. I. Pavlov, K. Bebenek, T. Matsuda, and T. A. Kunkel.** 2001. Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. Nature Immunol. **2:**537-541.
18.**Rosenberg, S. M.** 1997. Mutation for survival. Curr. Opinion Genet. Devel. **7:**829-834.
19.**Rosenberg, S. M., S. Longerich, P. Gee, and R. S. Harris.** 1994. Adaptive mutation by deletions in small mononucleotide repeats. Science. **265:**405-407.
20.**Rosenberg, S. M., C. Thulin, and R. S. Harris.** 1998. Transient and heritable mutators in adaptive evolution in the lab and in nature. Genetics. **148:**1559-1566.

21. **Scully, R., J. Chen, A. Plug, Y. Xiao, D. Weaver, J. Feunteun, T. Ashley, and D. M. Livingston.** 1997. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell. **88:**265-275.

22. **Wong, A. K. C., R. Pero, P. A. Ormonde, S. V. Tavtigian, and P. L. Bartel.** 1997. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. J. Biol. Chem. **272:**31941-31944.

23. **Zan, H., A. Komori, Z. Li, A. Cerutti, A. Schaffer, M. F. Flajnik, M. Diaz, and P. Casali.** 2001. The translesion DNA polymerase zeta plays a major role in Ig and *bcl*-6 somatic hypermutation. Immunity. **14:**643-653.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2002, p. 1535–1539 Vol. 46, No. 5
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.5.1535–1539.2002

NOTES

Chromosomal System for Studying AmpC-Mediated β-Lactam Resistance Mutation in *Escherichia coli*

Joseph F. Petrosino,[1]* Amanda R. Pendleton,[1]† Joel H. Weiner,[2] and Susan M. Rosenberg[1,3,4]*

Departments of Molecular and Human Genetics,[1] Biochemistry and Molecular Biology,[3] and Molecular Virology and Microbiology,[4] Baylor College of Medicine, Houston, Texas 77030-3411, and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7 Canada[2]

Received 5 September 2001/Returned for modification 25 November 2001/Accepted 9 February 2002

In some enterobacterial pathogens, but not in *Escherichia coli*, loss-of-function mutations in the *ampD* gene are a common route to β-lactam antibiotic resistance. We constructed an assay system for studying mechanism(s) of enterobacterial *ampD* mutation using the well-developed genetics of *E. coli*. We integrated the *Enterobacter ampRC* genes into the *E. coli* chromosome. These cells acquire spontaneous recombination- and SOS response-independent β-lactam resistance mutations in *ampD*. This chromosomal system is useful for studying mutation mechanisms that promote antibiotic resistance.

Mutation is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly (e.g., quinolone resistance mutations in genes encoding its *Escherichia coli* targets, *gyrA* and *gyrB* [16]). Other chromosomal mutations can cause mutator phenotypes that increase the likelihood of acquiring a resistance mutation (42). Mutations also ameliorate the otherwise deleterious effects on cell growth and physiology of some antibiotic resistance-conferring mutations (22). Although antibiotic resistance has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

In addition to spontaneous mutation in exponentially growing cells (growth-dependent mutation), other mutation mechanisms exist that may pertain to antibiotic resistance (34, 36, 37). For example, factors such as antibiotic concentration (23), environmental conditions (12), or other stress-inducing phenomena (1, 34, 36) may enhance or repress mutational machinery that leads to resistance mutations (for a review, see reference 31). Some mutation mechanisms or factors may be more important when the organism is under suboptimal growth conditions, as is probably the case during certain stages of an infection. In this study, we utilize a relatively well-described β-lactam resistance pathway as a model system to begin dissecting the mechanism(s) of antibiotic resistance mutation using the tools of *E. coli* genetics.

Chromosomally encoded AmpC β-lactamases confer β-lactam antibiotic resistance in many pathogenic and opportunistic bacteria and are ubiquitous in enterobacteria, except for the salmonellae, klebsiellae, *Proteus mirabilis*, *Shigella flexneri*, and *Shigella dysenteriae* (30, 32). Their expression is inducible in all but *E. coli* and the shigellae (30). In inducible strains, *ampC* transcription is controlled by the transcriptional activator AmpR (2). AmpR activity is regulated allosterically by two cell wall components, 1,6-anhydromuropeptide and UDP-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide). The first allows, and the second blocks, AmpR transcriptional activator activity at *ampC* (19). AmpD converts (activator-promoting) 1,6-anhydromuropeptide to (activator-blocking) UDP-MurNAc-pentapeptide, which then binds AmpR and blocks *ampC* transcription. Thus, loss-of-function mutations in *ampD* cause 1,6-anhydromuropeptide accumulation and constitutively induced AmpC β-lactamase production (7, 20, 21, 27). *ampD* missense and nonsense mutations are common in AmpC-mediated, β-lactam-resistant clinical isolates (25, 38). Also, some β-lactam antibiotics can induce expression of *ampC* by causing an increase in the cytoplasmic concentration of 1,6-anhydromuropeptide (7, 30).

E. coli lacks *ampR*, and low-level *ampC* expression results from a promoter embedded in the overlapping fumarate reductase (*frdABCD*) operon (13). High-level β-lactam resistance, mediated by *ampD* loss-of-function mutation, can be reconstituted in *E. coli* when the *ampR* and *ampC* genes of other enterobacteria are expressed from a plasmid (28, 35). We have integrated the *ampRC* genes from *Enterobacter cloacae* into the *E. coli* chromosome to assay *ampD* mutation, as selected by its β-lactam resistance phenotype. Background resistance imparted by the native *ampC* gene does not interfere with assays involving the reconstituted system. Integrating the *ampRC* genes into the chromosome improves upon previous plasmid-based *ampRC* expression systems by allowing genetic analyses not possible previously, first, because many mutant alleles used to study DNA repair, recombination, and mutation cause plasmid instability (e.g., reference 6). Second, the single-copy *ampRC* locus more closely models the situation in clini-

* Corresponding authors. Mailing address: Department of Molecular and Human Genetics, Baylor College of Medicine, Room S809A, Mail Stop BCM225, One Baylor Plaza, Houston, TX 77030-3411. Phone: (713) 798-6693. Fax: (713) 798-8967. E-mail for Joseph F. Petrosino: jp691032@bcm.tmc.edu. E-mail for Susan M. Rosenberg: smr@bcm.tmc.edu.

† Present address: Department of Cell Biology, Johns Hopkins School of Medicine, Baltimore, MD 21205.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
DM49	*lexA3*	29
FC526	Δ*recG263*::*kan*	11
GY8322	Δ(*srlR-recA*)*306*::Tn*10*	S. Sommer; ENZ280 (8) carrying the K5353 mini-F plasmid (9)
SMR821	*lexA3 malB*::Tn*9*	33
SMR1827	FC40 *sulA211*	33
SMR4562[a]	*rec*$^+$*att*λ$^+$	33; genotype identical to FC40 (4)
SMR4649	FC40 *sulA211 lexA51*	33
SMR5078	*recB21 recC22 sbcB15 sbcC201* $hsdr_K^-m_K^+$ (λ*xis1 cIts857*)	14
SMR5156	SMR4562 (λ*xis1 cIts857*)	SMR4562 × λSR446 (14)
SMR5201	*recB21 recC22 sbcB15 sbcC201* $hsdrK^-mK^+$ Δ*att*λ::*ampRC*	SMR5078 × DNA from pJP2
SMR5222	SMR4562 Δ*att*λ::*ampRC*	SMR 5156 × P1 SMR5201
SMR5225	SMR5222 Δ(*srlR-recA*)*306*::Tn*10*	SMR5222 × P1 GY8322
SMR5578	SMR5222 Δ*recG263*::*kan*	SMR5222 × P1 FC526
SMR5652	SMR5222 Δ*recG263*::*kan* Δ(*srlR-recA*)*306*::Tn*10*	SMR5578 × P1 GY8322
SMR5701	SMR1827 (λ*xis1 cIts857*)	SMR1827 × λSR446
SMR5702	SMR4649 (λ*xis1 cIts857*)	SMR4649 × λSR446
SMR5715	SMR5222 *lexA3 malB*::Tn*9*	SMR5222 × P1 SMR821
SMR5725	SMR5222 *sulA211 lexA51*	SMR5702 × P1 SMR5201
SMR5749	SMR5222 *sulA211*	SMR5701 × P1 SMR5201
Plasmids		
pEc1c	*E. cloacae ampRC*$^+$	35
pJP2	pTGV-light *ampRC*$^+$	This work
pJP19	pACYC184 *ampD*$^+$	This work[b]

[a] Full genotype is Δ(*lac-pro*)XIII *thi ara* Rif^r [F′ α45, *lacI*^{q} *lacI33*Ω*lacZ*].

[b] Plasmid pJP19, carrying the *E. coli ampD*$^+$ gene and promoter, was created by amplifying *ampD*$^+$ from *E. coli* wild-type strain MG1655 (3) chromosomal DNA using primers AmpD no. 1, 5′-GGGTTTTCATGAGAGGCGGCATGT TAAAACTCCAG-3′; and AmpD no. 2, 5′-GGGTTTAAGCTTTCATGTTGT CTCCTTGCTGACCAG-3′. The primers incorporate terminal *Bsp*HI and *Hin*dIII restriction sites at the 5′ and 3′ ends (respectively) of the amplified fragment. Amplified *ampD*$^+$ DNA and pACYC184 DNA (5) were digested with *Bsp*HI and *Hind*III, and the *ampD*$^+$ fragment was ligated into pACYC184. pJP19-mediated *ampD* expression was confirmed by complementation to β-lactam sensitivity of four independent *ampD* β-lactam-resistant mutants.

cally relevant enterobacterial resistance (30). Third, this chromosomal system excludes mutations that confer β-lactam resistance by increasing plasmid (and, therefore, *ampC*) copy number.

An *ampRC* expression cassette in the *E. coli* chromosome was constructed as follows. The strains and plasmids used are shown in Table 1. SMR5222, an *E. coli* strain carrying the *E. cloacae ampRC*$^+$ genes in the *E. coli* chromosome, was constructed using the TGV (transgenic vector) system for integrating linear DNA cassettes into the *E. coli* chromosome (14). *E. cloacae* strain MHN1 *ampRC*$^+$ genes were isolated from plasmid pEc1c (35) by digestion with *Bam*HI and *Sal*I and were ligated into *Bgl*II- and *Xho*I-digested pTGV-light (14) plasmid DNA, creating pJP2. pJP2 was digested with *Nde*I to generate an *ampRC*$^+$ fragment flanked on both sides by homology to the *E. coli* attachment site for phage λ (*att*λ) for linear transformation in the TGV system (14). SMR5201, a transformant carrying *ampRC*$^+$ replacing *att*λ (confirmed by PCR as described elsewhere [14]) was used as a P1 donor to move *att*λ::*ampRC*$^+$ into SMR5156 by P1 transduction (as described elsewhere [14]) to create SMR5222. Subsequent strains are isogenic to SMR5222 and were created using standard phage P1 transduction (referenced elsewhere [14]), and the constructions are outlined in Table 1.

We used the chromosome-based *ampRC* expression system to determine rates of spontaneous (growth-dependent) ampicillin resistance (Amp^r) mutation in otherwise isogenic strains

TABLE 2. Rates of β-lactam resistance mutation in *E. coli* DNA repair-deficient mutants

Relevant genotype[a] and expt[b]	Mutation rate (mutations/cell/generation, 10^{-7})	Rate relative to *rec*$^+$ within each expt	Mean mutation rate (mutations/cell/generation, 10^{-7}) ± SE	Mean relative to *rec*$^+$ within each expt ± SE
rec$^+$				
1	0.460	1	1.4 ± 0.3	1.0
2	2.18	1		
3	2.71	1		
4	1.10	1		
5	1.60	1		
6	0.632	1		
7	1.20	1		
recA			1.0 ± 0.3	0.66 ± 0.1
1	0.242	0.53		
2	0.908	0.42		
3	2.72	1.0		
4	0.693	0.63		
5	0.950	0.59		
6	0.277	0.44		
7	1.24	1.0		
recG			2.0 ± 0.8	1.0 ± 0.2
1	0.342	0.74		
2	3.20	1.5		
3	2.38	0.88		
recA recG			1.2 ± 0.5	0.65 ± 0.1
1	0.235	0.51		
2	1.70	0.78		
3	1.79	0.66		
lexA3			0.96 ± 0.2	0.79 ± 0.1
5	1.48	0.93		
6	0.362	0.57		
7	1.05	0.88		
sulA			1.0 ± 0.1	0.93 ± 0.1
5	1.05	0.66		
6	0.665	1.1		
7	1.29	1.1		
sulA lexA5 (Def)			1.7 ± 0.3	1.5 ± 0.04
5	2.45	1.5		
6	0.895	1.4		
7	1.69	1.4		

[a] Strains used were SMR5222, *rec*$^+$ (recombination and SOS response proficient); SMR5225, *recA* (recombination and SOS response deficient [40]); SMR5578, *recG* (recombination proficient, elevated for stationary-phase-mutation [11, 15]); SMR5652, *recG recA* (recombination, SOS, and stationary-phase mutation deficient [11, 15]); SMR5715, *lexA3* (recombination proficient, SOS gene induction defective [40]); SMR5749, *sulA* (allows viability in the presence of a *lexA*-null mutation [40]); and SMR5725, *sulA lexA51*(Def) (SOS-induced genes expressed constitutively [40]).

[b] Data preceded by the same experiment number were gathered in parallel.

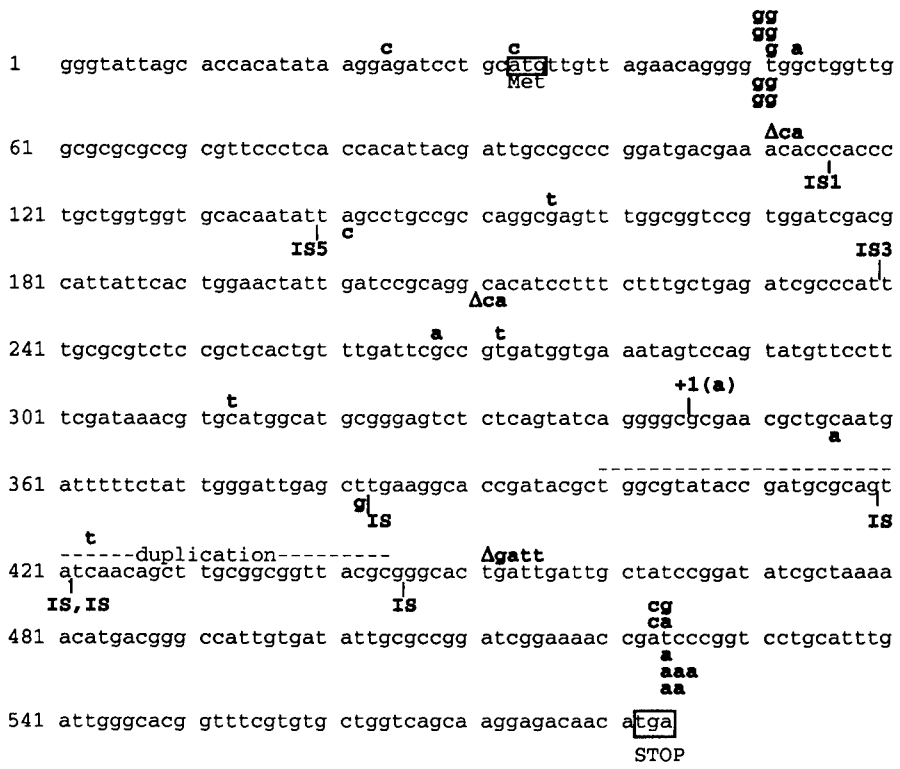

FIG. 1. Location of *ampD* mutations. Forty independent Amp^r mutants (20 from *rec*$^+$ strain SMR5222 and 20 from *recA* strain SMR5225) were screened for *ampD* mutations by complementation to β-lactam sensitivity using pJP19 (Table 1). Apparent *ampD* mutations were sequenced (Lone Star Labs, Houston, Tex.) from PCR products using primers AmpD no. 3, 5′-GCGCGTCTCCGCTCACTGTTT-3′; and AmpD no. 4, 5′-GCA TGCCATGCACGTTTATCG-3′. The PCR products were generated with primers AmpD no. 1 and AmpD no. 2 (legend to Table 1). The *E. coli ampD* sequence is given, along with 32 bp upstream of the ATG start codon. Mutations found in *rec*$^+$ mutants are indicated above, and mutations found in *recA* mutants are shown below, the sequence. IS, insertion sequence.

lacking various recombination and SOS genes. Growth-dependent mutation rates were measured in 15 tube fluctuation tests. Fifteen independent cultures for each strain were grown to saturation in 5 or 10 ml of Mueller-Hinton (MH) broth (Difco), shaking at 37°C. Cultures were diluted 10-fold, and 50 μl was plated in duplicate on MH agar plates containing 100 μg/ml ampicillin. The plates were incubated overnight at 37°C, and Amp^r colonies were counted. Viable cell counts of the saturated cultures were from dilutions plated on MH incubated overnight at 37°C. Mutation rates were calculated by the method of the median (26).

The recombination and SOS genes examined—*recA*, *recG*, *recA recG*, *lexA3*(Ind$^-$), *sulA*, and *sulA lexA*(Def)—are not required for most growth-dependent mutation; however, they are required for a mechanism of mutation observed under growth-limiting conditions of carbon starvation (for a review, see reference 37; see also references 41 and 43). Moreover, the SOS response controls several mutation-promoting proteins (40) whose possible involvement in β-lactam resistance mutation we wished to test. The β-lactam resistance mutation rate in recombination- and SOS-proficient (*rec*$^+$) cells is about 1.4×10^{-7} cell^{-1} generation^{-1} (Table 2). The strains tested displayed only small differences in growth-dependent mutation rates, indicating that recombination and SOS genes are not important for most growth-dependent β-lactam resistance mutation (Table 2).

The following experiments demonstrated that the β-lactam resistance mutations are in *ampD*. Based on prior observations in an *E. coli* model and in clinical isolates of enterobacterial pathogens (7, 27), we expected most of the β-lactam resistance mutations to be in *ampD*. To test this, 20 independent ampicillin resistant mutants (each from a separate independent culture) from the *rec*$^+$ and *recA* fluctuation test experiments were examined further. β-Lactam sensitivity was restored to each of the 40 mutants by transforming each with pJP19 (Table 1) carrying the *ampD*$^+$ gene. This complementation test shows that the ampicillin resistance mutations of the 40 independent mutants are recessive, loss-of-function mutations in *ampD*.

We determined the *ampD* sequences for each of the 40 mutants. Little difference was found between the *rec*$^+$ and *recA* backgrounds (Fig. 1), suggesting similar mutation mechanism(s) in each. Many different mutations and insertions in *ampD* conferred AmpC-mediated β-lactam resistance.

Among the eight substitution mutations identified here, two were identified previously from β-lactam resistant isolates. A Trp7Gly substitution in AmpD occurred in both the *rec*$^+$ and *recA* strains (Fig. 2) and previously in *E. coli* ampicillin-resistant mutants of cells expressing the *E. cloacae ampRC* genes from a plasmid (18). Also, we found Asp164Glu and Asp164Ala in both *rec*$^+$ and *recA*. Asp164Glu was found previously in *Citrobacter freundii* (39). Both previous *ampD* mutations were shown to cause full derepression of *ampC*, as *ampD*-null mutations do (28, 39).

Other *ampD* substitution mutations found include

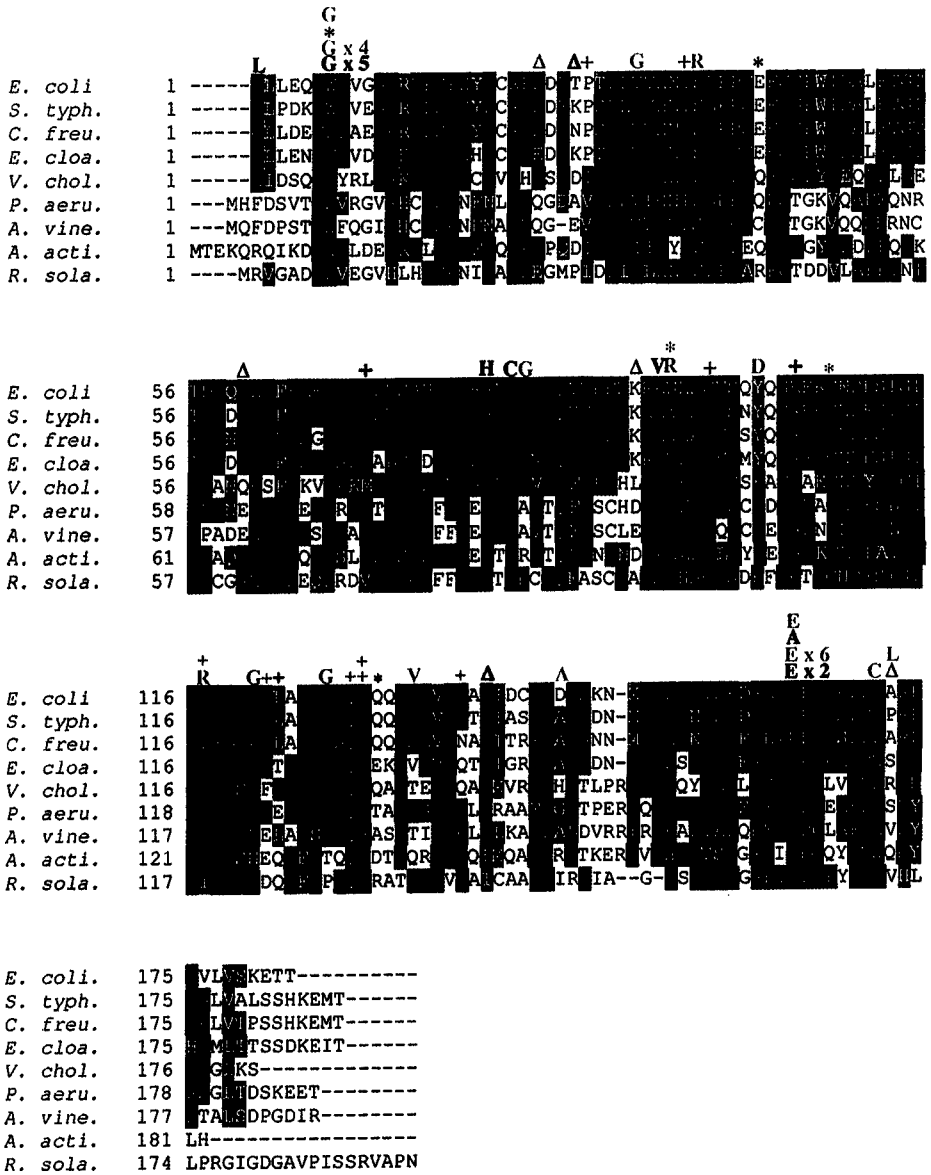

FIG. 2. Amino acid substitutions, insertions, and deletions identified in the *rec*$^+$ and *recA* mutants aligned with the *ampD* genes from nine enterobacteria. Also included are previously identified *ampD* mutant proteins from other laboratories (10, 18, 24, 28). *rec*$^+$ mutations are in black, *recA* mutations are in red, and previously identified mutations are in blue. Insertions are indicated by a plus sign (+), deletions are indicated by a delta (Δ), and nonsense mutations are indicated by an asterisk (*). Mutations isolated multiple times show the number of times that each was encountered for each strain (*rec*$^+$ or *recA*). Abbreviations: *S. typh*, *Salmonella enterica* serovar Typhimurium; *C. freu*, *C. freundii*; *E. cloa*, *E. cloacae*; *V. chol*, *Vibrio cholerae*; *P. aeru*, *Pseudomonas aeruginosa*; *A. vine*, *Azotobacter vinelandii*; *A. acti*, *Actinobacillus actinomycetemcomitans*; and *R. sola*, *Ralstonia solanacearum*. Alignment was performed using ClustalW (17) and formatted using BOXSHADE.

Ser37Arg, Arg80His, Gly82Cys, Ala94Val, and Leu117Arg (Fig. 2). Although these might or might not inactivate *ampD* fully, substitutions that alter or abolish AmpD function reveal amino acids that are important for AmpD structure and/or function. Conservative substitutions, such as Ala94Val or Asp164Glu, highlight the specific steric and/or chemical requirements of the wild-type amino acids. For example, the intolerance for Ala94Val suggests that the smaller size of alanine is important here, because both amino acids are similarly hydrophobic. The Asp164Glu substitution involves similar charges, suggesting that this amino acid position makes important catalytic or structural contacts disrupted by the larger glutamic acid side chain. Asp164 is probably not simply a surface amino acid, because it has a seemingly stringent size requirement and because alanine at this position is also not tolerated.

Alignments show that *ampD* is highly conserved among various bacteria (e.g., Fig. 2). Asp164, Ala94, and the other substituted amino acids from our mutation studies are highly conserved among the aligned *ampD* sequences (Fig. 2) and further highlight their potential structural and/or functional importance.

The variety of loss-of-function mutations observed in this system suggests its utility for studying many kinds of mutation

mechanisms. This system may be useful additionally for studying the forward mutation spectra caused by potential damaging agents and environmental factors, because mutations in the small *ampD* gene are easily selected and sequenced.

We thank Mellanie Price for excellent technical support and Mary-Jane Lombardo, P. J. Hastings, Gregory McKenzie, Megan Hersh, Jeanine Pennington, and Rebecca Ponder for critical comments on the manuscript.

This work was supported by a Department of Defense Breast Cancer Research Postdoctoral Fellowship (to J.F.P.) and National Institutes of Health Grant R01-AI43917.

REFERENCES

1. **Alonso, A., E. Campanario, and J. L. Martinez.** 1999. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. Microbiology **145**:2857–2862.
2. **Bartowsky, E., and S. Normark.** 1991. Purification and mutant analysis of *Citrobacter freundii* AmpR, the regulator for chromosomal AmpC beta-lactamase. Mol. Microbiol. **5**:1715–1725.
3. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glassner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453–1474.
4. **Cairns, J., and P. L. Foster.** 1991. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics **128**:695–701.
5. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
6. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167**:327–335.
7. **Dietz, H., D. Pfeifle, and B. Wiedemann.** 1997. The signal molecule for β-lactamase induction in *Enterobacter cloacae* is the anhydromuramyl-pentapeptide. Antimicrob. Agents Chemother. **41**:2113–2120.
8. **Dri, A. M., J. Rouviere-Yaniv, and P. L. Moreau.** 1991. Inhibition of cell division in *hupA hupB* mutant bacteria lacking HU protein. J. Bacteriol. **173**:2852–2863.
9. **Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista, G. C. Walker, and R. Devoret.** 1989. New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. **171**:2415–2423.
10. **Ehrhardt, A. F., C. C. Sanders, J. R. Romero, and J. S. Leser.** 1996. Sequencing and analysis of four new *Enterobacter ampD* alleles. Antimicrob. Agents Chemother. **40**:1953–1956.
11. **Foster, P. L., J. M. Trimarchi, and R. A. Maurer.** 1996. Two enzymes, both of which process recombination intermediates, have opposite effects on adaptive mutation in *Escherichia coli*. Genetics **142**:25–37.
12. **Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman, M. Fons, and F. Taddei.** 2001. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science **291**:2606–2608.
13. **Grundström, T., and B. Jaurin.** 1982. Overlap between *ampC* and *frd* operons on the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA **79**: 1111–1115.
14. **Gumbiner-Russo, L. M., M.-J. Lombardo, R. G. Ponder, and S. M. Rosenberg.** 2001. The TGV *t*ransgenic *v*ectors for single-copy gene expression from the *Escherichia coli* chromosome. Gene **273**:97–104.
15. **Harris, R. S., K. J. Ross, and S. M. Rosenberg.** 1996. Opposing roles of the Holliday junction processing systems of *Escherichia coli* in recombination-dependent adaptive mutation. Genetics **142**:681–691.
16. **Herrera, G., V. Aleixandra, A. Urios, and M. Blanco.** 1993. Quinolone action in *Escherichia coli* cells carrying *gyrA* and *gyrB* mutations. FEMS Microbiol. Lett. **106**:187–191.
17. **Higgins, D. G., J. D. Thompson, and T. J. Gibson.** 1996. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. **266**:383–402.
18. **Honoré, N., M.-H. Nicolas, and S. T. Cole.** 1989. Regulation of enterobacterial cephalosporinase production: the role of the membrane-bound sensory transducer. Mol. Microbiol. **3**:1121–1130.
19. **Jacobs, C., J.-M. Frère, and S. Normark.** 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell **88**:823–832.
20. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13**:4684–4694.
21. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. Van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, and J.-M. Frère.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15**:553–559.
22. **Karunakaran, P., and J. Davies.** 2000. Genetic antagonism and hypermutability in *Mycobacterium smegmatis*. J. Bacteriol. **182**:3331–3335.
23. **Kohler, T., M. Michea-Hamzehpour, P. Plesiat, A. L. Kahr, and J. C. Pechère.** 1997. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41**:2540–2543.
24. **Kopp, U., B. Wiedemann, S. Lindquist, and S. Normark.** 1993. Sequences of wild-type and mutant *ampD* genes of *Citrobacter freundii* and *Enterobacter cloacae*. Antimicrob. Agents Chemother. **37**:224–228.
25. **Langaee, T. Y., L. Gagnon, and A. Huletsky.** 2000. Inactivation of the *ampD* gene in *Pseudomonas aeruginosa* leads to moderate-basal-level and hyperinducible AmpC β-lactamase expression. Antimicrob. Agents Chemother. **44**: 583–589.
26. **Lea, D. E., and C. A. Coulson.** 1949. The distribution of the numbers of mutants in bacterial populations. J. Genet. **49**:264–285.
27. **Lindberg, F., S. Lindquist, and S. Normark.** 1987. Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. J. Bacteriol. **169**:1923–1928.
28. **Lindquist, S., M. Galleni, F. Lindberg, and S. Normark.** 1989. Signaling proteins in enterobacterial AmpC β-lactamase regulation. Mol. Microbiol. **3**:1091–1102.
29. **Little, J. W., S. H. Edmiston, L. Z. Pacelli, and D. W. Mount.** 1980. Cleavage of the *Escherichia coli* LexA protein by the RecA protease. Proc. Natl. Acad. Sci. USA **77**:3225–3229.
30. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8**:557–584.
31. **Martinez, J. L., and F. Baquero.** 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. **44**:1771–1777.
32. **Maurelli, A. T., R. E. Fernández, C. A. Bloch, C. K. Rode, and A. Fasano.** 1998. "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*. Proc. Natl. Acad. Sci. USA **95**:3943–3948.
33. **McKenzie, G. J., R. S. Harris, P. L. Lee, and S. M. Rosenberg.** 2000. The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. USA **97**:6646–6651.
34. **McKenzie, G. J., and S. M. Rosenberg.** 2001. Adaptive mutations, mutator DNA polymerases and genetic change strategies of pathogens. Curr. Opin. Microbiol. **4**:586–594.
35. **Nicolas, M.-H., N. Honore, V. Jarlier, A. Philippon, and S. T. Cole.** 1987. Molecular genetic analysis of cephalosporinase production and its role in β-lactam resistance in clinical isolates of *Enterobacter cloacae*. Antimicrob. Agents Chemother. **31**:295–299.
36. **Riesenfeld, C., M. Everett, L. J. Piddock, and B. G. Hall.** 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrob. Agents Chemother. **41**:2059–2060.
37. **Rosenberg, S. M.** 2001. Evolving responsively: adaptive mutation. Nat. Rev. Genet. **2**:504–515.
38. **Sanders, C. C., and W. E. Sanders, Jr.** 1992. β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis. **15**:824–839.
39. **Stapleton, P., K. Shannon, and I. Phillips.** 1995. DNA sequence differences of *ampD* mutants of *Citrobacter freundii*. Antimicrob. Agents Chemother. **39**:2494–2498.
40. **Sutton, M. D., B. T. Smith, V. G. Godoy, and G. C. Walker.** 2000. The SOS response: recent insights into *umuDC*-dependent mutagenesis and DNA damage tolerance. Annu. Rev. Genet. **34**:479–497.
41. **Taddei, F., J. A. Halliday, I. Matic, and M. Radman.** 1997. Genetic analysis of mutagenesis in aging *Escherichia coli* colonies. Mol. Gen. Genet. **256**:277–281.
42. **Taddei, F., I. Matic, B. Godelle, and M. Radman.** 1997. To be a mutator, or how pathogenic and commensal bacteria can evolve rapidly. Trends Microbiol. **5**:427–428.
43. **Taddei, F., I. Matic, and M. Radman.** 1995. cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. Proc. Natl. Acad. Sci. USA **92**:11736–11740.